Bioresource Technology 181 (2015) 90–96

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Wastewater treatment and biodiesel production by *Scenedesmus obliquus* in a two-stage cultivation process

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HIGHLIGHTS

- Wastewater treatment with microalgae produced biomass to be used as biodiesel source.
- Wastewater cultured *S. obliquus* increased lipid content in response to stress factors.
- CO_2, light and salt factors acting isolated increased lipids.
- Salt presence in darkness increased lipids avoiding the use of photobioreactors.
- ω-3 eicosapentaenoic acid content of biomass slightly exceeded EU biodiesel normative.

ARTICLE INFO

Article history:
Received 23 October 2014
Received in revised form 5 January 2015
Accepted 6 January 2015
Available online 17 January 2015

Keywords:
Scenedesmus obliquus
Wastewater
Biodiesel
Lipid
Fatty acid

ABSTRACT

The microalga *Scenedesmus obliquus* was cultured in two cultivation stages: (1) in batch with real wastewater; (2) maintaining the stationary phase with different conditions of CO_2, light and salinity according to a factorial design in order to improve the lipid content. The presence of the three factors increased lipid content from 35.8% to 49% at the end of the second stage; CO_2 presence presented the highest direct effect increasing lipid content followed by light presence and salt presence. The ω-3 fatty acids content increased with CO_2 and light presence acting in isolation, nevertheless, when both factors acted together the interaction effect was negative. The ω-3 eicosapentaenoic acid content of the oil from *S. obliquus* slightly exceeded the 1% maximum to be used as biodiesel source (EU normative). Therefore, it is suggested the blend with other oils or the selective extraction of the ω-3 fatty acids from *S. obliquus* oil.

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1. Introduction

Microalgae cultivation is currently been proposed as promising source of biofuels and high-value products as nutraceuticals (Borowitzka, 2013). However, microalgae cultivation in a commercial scale for biofuel production appears not to be economically feasible and sustainable (Markou and Nerantzis, 2013). On the other hand, nitrogen and phosphorus present in wastewater could present a cheap raw material for microalgae cultivation making the production of microalgae as by-product of the wastewater treatment as a niche opportunity (Park et al., 2011). In terms of environmental benefits, wastewater microalgal cultivation for subsequent biofuel production implies a reduction in carbon and water footprint that increases the sustainability of the biofuel from algae production (Clarens et al., 2010).

Microalgae cultivated under stress conditions have the ability to alter their biomass composition and accumulate lipids and carbohydrates which could be used for biofuel production, therefore their potential to be used as biofuel feedstock increases (Markou and Nerantzis, 2013). Under optimal conditions of growth, algae synthesize fatty acids to produce membrane polar lipids, as glycolipids and phospholipids, while under stress conditions many algae alter their lipid synthesis pathways and accumulate neutral lipids, mainly in the form of triacylglycerols. These latter lipids do not perform a structural role as the first ones but serve as a carbon and energy storage. These triacylglycerol lipids can be extracted and isolated from harvested microalgae and then converted to biodiesel by transesterification (Hu et al., 2008). The fatty acid profile of the microalgal oil plays a crucial role in the performance of biodiesel properties. These critic parameters that vary according to the carbon chain sizes and the disposition of double bonds are cetane number (*CN*), iodine value (*IV*) and cold filter plugging point (*CFPP*) (Knothe, 2005; Ramos et al., 2009).

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http://dx.doi.org/10.1016/j.biortech.2015.01.018
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Nutrient starvation during stationary growth phase is well known as lipid enhancer, in this sense there are other factors as temperature, light, salinity and growth phase that have been shown to influence the algal metabolism (Boyle and Morgan, 2009). This lipid accumulation occurs at the expense of energy used for growth, leading to a decrease in growth rate and thus in biomass and lipid productivity (Wijffels and Barbosa, 2010). Cultivation in multiple-stage process is suggested to avoid the hamper of productivity reduction. Several authors propose a first stage for biomass growth in optimum conditions followed by the application of subsequent stress conditions promoting lipid accumulation (Prathima Devi et al., 2012; Markou and Nerantzis, 2013).

The species *Scenedesmus obliquus* has been commonly proposed as a candidate strain to treat wastewaters (Arbib et al., 2013; Ruiz et al., 2014). Moreover this species can accumulate quantities of lipids under stress conditions as nitrogen deficiency (Mandal and Mallick, 2009) and presents an adequate fatty acid profile in terms of linolenic acid and polyunsaturated acids to produce biodiesel (Gouveia and Oliveira, 2009).

Considering that the energetic requirements of microalgae production processes can determine the sustainability of the whole process, it becomes mandatory to apply the stress factors in a second cultivation stage with minimum energy consumption. Under our knowledge there are no previous works describing the influence of stress factors on microalgae biomass once the objective of the wastewater treatment is achieved. For this study there have been selected 3 stress factors to be applied, these are (1) the light presence, which is determined by the maintenance of the culture in the photobioreactors or by transferring the cultures to an opaque liquid container; (2) the salinity, which can be easily increased by the addition of available and cheap resources as NaCl or marine water; and finally (3) the use of CO_2, which is an already required resource in microalgae cultures.

In order to study the effect of these factors on lipid content and lipid profile of microalgae biomass, on a second cultivation stage subsequent to a wastewater treatment, a full factorial design based on the presence or absence of light, salinity and CO_2 has been applied to cultures of *S. obliquus* in the stationary phase after a batch growth in real wastewater. The design of the experiments included the effect of lipid enhancer factors as the nutrient starvation and the aging of the cultures.

2. Methods

2.1. Microorganism

S. obliquus (SAG 276.10) was obtained from Sammlung von Algenkulturen, pflanzenphysiologisches Institut, (Universität Göttingen, Germany). Stock cultures were maintained routinely in liquid nutritive COMBO medium by regular subculturing at 2-weeks intervals. Cultures were maintained at 20 ± 1 °C temperature and 143 μmol/m^2 s PAR light intensity under 14/10 light/dark cycle.

2.2. Wastewater

The feedstock used was secondarily pre-treated wastewater (WW) from the wastewater treatment plant "El Torno" located in Chiclana de la Frontera in southern Spain (municipality of around 80,000 inhabitants) (36°25′37.340″N, −6°9′23.386″W). The effluent was collected after the preliminary screening, primary sedimentation, activated sludge and secondary sedimentation processes. The WW was filtered by 1 μm nominal pore glass fiber filter previous to be used as culture medium, and characterized as follows: 20.09 ± 1.3 mg Total-N/L, 15.79 ± 0.07 mg NH_4-N/L, 2.17 ± 0.02 mg NO_2-N/L, 0.24 ± 0.01 mg NO_3-N/L, 1.89 mg N/L (organic nitrogen), 1.55 ± 0.01 mg Total-P/L, 10.67 ± 0.33 mg C/L (total organic carbon), 70 ± 2.1 mg O_2/L (chemical oxygen demand) and pH = 9.27. Dissolved species of N were determined by ionic chromatography (Metrohm, 881 Compact IC pro Anion and MCS 882 Compact IC plus Cation). Total N and P were determined as explained in the nutrient determination section and organic N was calculated by the subtraction of the N inorganic species from total concentration. Total organic carbon was determined by means of high temperature catalytic oxidation in an analyzer (Shimadzu TOC-5050A), chemical oxygen demand was determined with Spectroquant® COD test kits (Merck, 1.14541.0001) and pH was measured with a pH meter (Crison, GLP 21).

2.3. Experimental set-up

Experiments were conducted in batch by using 2000 mL borosilicate cylindrical flasks as photobioreactors (12.5 cm diameter and 14.5 cm height). Illumination was provided from the top of the flasks by using eight fluorescent lamps (four PHILIPS Master TLD 58 W/840 and four SYLVANIA Gro-Lux F 58 W/GRO-T8) with 143 μmol m^{-2} s^{-1} PAR light intensity and 14/10 light/dark cycle. PAR light intensity was measured by a digital light meter (Hansatech QRT1 Quantitherm light meter). 1.5 L of wastewater was inoculated with 90 mL suspension of pre-cultured cells, to obtain an initial biomass concentration in all reactors of around 0.1 g/L (biomass dry weight). The experiments were conducted at (20 ± 1 °C) in a thermostatic chamber. Aeration was supplied from the bottom of the flask at a flow rate of 1.5 L/min and enriched with CO_2 at 4%, this value is tolerated by this species (Tang et al., 2011) and simulates the flue gas of a natural gas combined heat and power plant (Shao et al., 1995).

2.4. Experimental design

To study the effects of salinity, CO_2 and light to already grown biomass, a replicated multilevel factorial design which included all the possible combinations among these three factors was applied (Table 1).

This factorial design was employed to screen factors that may have significant effects on response(s). Predefined values of the factors are expressed in terms of levels. The quality of the results from the factorial design was evaluated using a factorial analysis

Table 1
Experimental domain, (−) and (+) are codes of the factorial experimental design meaning absence and presence, respectively, of CO_2 (C), salinity (S) and light (L) factors.

Experiment	Codification	CO_2 (% in aeration)	Salinity (g/L marine salt)	Light
1	E1 (+C+L+S)	4	15	Presence
2	E2 (+C−L+S)	4	0	Presence
3	E3 (+C+L−S)	4	15	Absence
4	E4 (+C−L−S)	4	0	Absence
5	E5 (−C+L+S)	0	15	Presence
6	E6 (−C−L+S)	0	0	Presence
7	E7 (−C+L−S)	0	15	Absence
8	E8 (−C−L−S)	0	0	Absence

of the variance (ANOVA). This test determines the presence of significant differences between groups of data within a variable and allows discrimination of factors that significantly affected experimental results. If results are affected by the interaction of various factors, only the interaction effect should be considered and not the factors independently (Scheffé, 1959). All statistical analyses were performed with the STATISTICA software (Version 7.0, StatSoft, 2004).

Seventeen reactors containing *S. obliquus* in wastewater were carried in batch until stationary growth phase in a first stage of 400 h. Then, in a second stage, the three factors were combined as stated in Table 1. Nitrogen and phosphorus content in the wastewater were determined at the end of the first stage (400 h) giving values below of the detection limit; therefore, at the second stage all reactors were nutrient starved. The CO_2 factor was studied by maintaining or not the enrichment of CO_2 (4%) in the air bubbled into the photobioreactors. The salinity factor was studied by adding or not, natural marine salt until a concentration of 15 g/L. Marine salt was chosen as reactive due to its content in different compounds that might be useful for microalgae metabolism. Characterization of the natural marine salt was <0.025 mg As/kg, <0.05 mg Hg/kg, <0.15 mg Pb/kg, <0.2 mg Cd/kg, <2 mg Cu/kg, <15 mg NO_3/kg, 0.07 mg NO_2/kg, 0.12 mg NH_4/kg, 97.6% NaCl, 10 mg/kg of Iron Salts, 0.59 g/kg of Calcium Salts and 1.27 mg I/kg. The light factor was studied by allowing or restricting the light penetration into photobioreactors through an opaque film.

The factorial design was set up in a simultaneous double run of 8 experiments plus a control experiment that was monitored before the beginning of the second stage; its biomass was taken as control of lipid content and relative fatty acid abundance.

2.5. Biomass monitoring

Daily biomass growth was periodically evaluated (at least every 24 h) through the correlation (Eq. (1)) between optical density (DO_{680}) measured at 680 nm in a spectrophotometer (Thermo GENESYS 10-Vis) and the dry weight of algal biomass determined gravimetrically as suspended solids (SS) according to the standardized method 2540-D (APHA–AWWA–WPCF, 1992).

$$\mathrm{SS}\ (\mathrm{g/L}) = 0.4186 * \mathrm{DO}_{680} - 0.0214 \quad R^2 = 0.9901 \tag{1}$$

2.6. Total lipid determination

The total lipids (*TL*) were extracted according to the modified method reported by (Takagi et al., 2006). 90 mg of the lyophilized biomass pellet was covered with solvents, 12 ml of 2:1 trichloromethane/methanol mixture and 0.6 g of analytical grade quartz (SIGMA particle size 10–30 μm) (Wiltshire et al., 2000). Samples were subsequently placed in an ultrasound bath (35 kHz; 80 W) and sonicated for 90 min. The original solvent was collected and extraction was repeated. Final extracts were centrifuged at 5000 rpm and filtered to ensure solids separation. Then, the solvents were removed under vacuum in a rotary evaporator at 65 °C. The remainder was dried in a desiccator for 24 h, and weighed as the total lipid. All extractions were done in triplicate.

2.7. Fatty acid determination

Transesterification of fatty acids (*FA*) into *FA* methyl esters (*FAME*) was carried out as follows. 1 mL of hexane was added to dissolve evaporated lipid extracts, then 100 mg lipid extracts were added with 2 mL of n-heptane and shacked, then 0.2 mL of methanolic solution of KOH (2 N) was added and vigorously vortexed for 30 s. After clarification, the upper heptane layer was analyzed by gas chromatography. 2 μL of each sample were injected in a gas chromatograph (Hewlett Packard HP 5890 Series GC) equipped with a flame ionization detector and a capillary column (60 m × 0.25 mm × 0.2 μm) (SUPELCO SP-2380). *FAME* were identified by comparing its retention times and peak areas with pure standards (Sigma Chemicals).

2.8. Estimation of biodiesel properties based on FAME profiles

The critical parameters that determine the quality of the biodiesel were estimated in relation to the molecular structures of *FAME*, which may vary according to carbon chain sizes and the amount and/or position of double bonds. Biodiesel quality in Europe is defined by the European Standard EN 14214. This normative establishes critical biodiesel parameter limitations to be satisfied by the biodiesel generated from vegetable oil. Some of these parameters depend on the refinement, the transesterification process and the quality of purification step while others depend on the fatty acid profile of the parent vegetal oil, these critic parameters depending on oil fatty acids profile are cetane number (*CN*), iodine value (*IV*) and cold filter plugging point (*CFPP*). They vary according to the concentration of each fatty acid, the carbon chain sizes and the disposition of double bonds (Knothe, 2005; Ramos et al., 2009). Furthermore, several models have been developed to predict these critical biodiesel properties from the fatty acid composition of the oil (Stansell et al., 2012). Therefore for the *CN* the model was calculated as stated in Stansell et al. (2012) using the following equations where "*n*" is the carbon number and "*db*" is the number of double bonds.

CN of saturated *FA* (*SFA*):

$$CN = -107.71 + 31.126\ n - 2.042\ n^2 + 0.0499\ n^3 \tag{2}$$

CN of monounsaturated *FA* (*MUFA*)

$$CN = 109 - 9.292\ n + 0.354\ n^2 \tag{3}$$

CN of polyunsaturated *FA* (PUFA)

$$CN = -21.157 + (7.965 - 1.785\ db + 0.235\ db^2)\ n - 0.099\ n^2 \tag{4}$$

Then, the *CN* of the biodiesel is given by the following equation where "CN_i" is the *CN* of each *FAME* and "mp_i" is the mass percentage of each *FAME*:

$$CN = 1.068 \sum (CN_i * m_i) - 6.747 \tag{5}$$

The *IV* was calculated as stated in Nascimento et al. (2013) where "m_i" is the molecular mass of each *FAME*:

$$IV = \sum (254 * db_i * mp_i)/m_i \tag{6}$$

The *CFPP* was calculated as stated in Nascimento et al. (2013) with the following equation:

$$CFPP = (3.1417 * LCSF) - 16.477 \tag{7}$$

where *LCSF* is the long-chain saturated factor and is calculated by weighing up values of the longer chains (C16, C18, C20, C22 and C24 are the *mp* of each *FA*):

$$LCSF = (0.1 * mp_{C16}) + (0.5 * mp_{C18}) + (1 * mp_{C20}) + (1.5 * mp_{C22}) + (2 * mp_{C24}) \tag{8}$$

3. Results and discussion

3.1. Biomass

Fig. 1 shows the biomass evolution in the first and second stages of the cultures of *S. obliquus*.

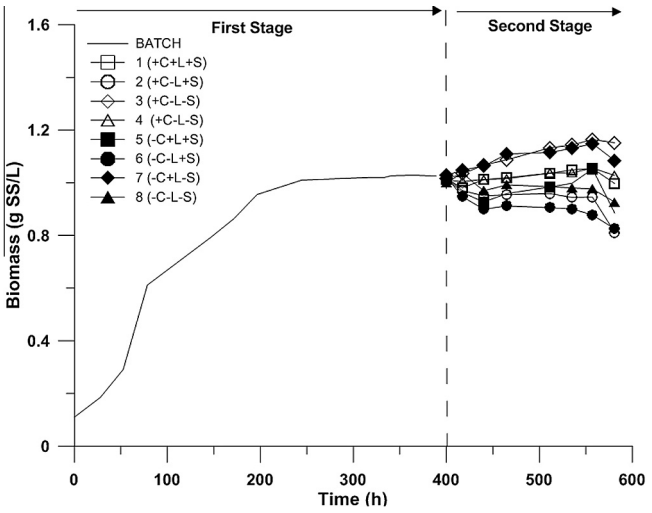

Fig. 1. Biomass evolution in the first and second stages of the experimentation.

As can be observed in Fig. 1, *S. obliquus* reached 1.03 g/L at the end of the first stage. At the second stage, no later growth occurred, only slightly increases and decreases of the biomass concentration that varied between 0.811 and 1.15 g/L. Similar biomass results were obtained by *Scenedesmus acutus* cultured in municipal wastewater by Sacristán de Alva et al. (2013). The initial pH of the cultures at the beginning of the second stage was 8.19 ± 0.29. Then, in the CO_2 presence experiments the pH stabilized around 7.5 and in the non CO_2 presence experiments around 8.5 (data not shown).

3.2. Total lipid

Fig. 2 shows the total lipid content of the biomass of each culture at the second stage.

The *TL* at the end of the first stage was 35.8%. This result is higher than others reported elsewhere under nutrient replete conditions, i.e. Griffiths and Harrison (2009) calculated a *TL* of 21% of *S. obliquus* averaging from literature. These authors (Griffiths and Harrison, 2009) reported similar values to that obtained at the end of the first stage for *S. obliquus* grown under nitrogen deficient conditions. Therefore, it can be stated that the lipid synthesis related to nitrogen deficiency started before the end of the first stage. The *TL* obtained here is in accordance to the results presented by Chen et al. (2012) for a *Scenedesmus* sp. microalgae cultured during 36 days in a synthetic medium. These authors described the accumulation of neutral lipids (triacylglicerols) coupled with nitrate at very low levels in the medium.

The experiments that presented higher *TL* at the end of the second stage with respect to the end of the first stage were: E1 (+C+L+S) (the highest), E2 (+C−L+S), E3 (+C+L−S), E4 (+C−L−S) and E5 (−C+L+S); and in percentage were 37.1%, 19.6%, 17.7%, 6.3% and 9.4%, respectively. The experiments that decreased the *TL* were E6 (−C−L+S), E7 (−C+L−S) and E8 (−C−L−S) implying a decrease with respect to the first stage of 7.5%, 2.9% and 9.8%, respectively.

The factor combination that promoted the highest *TL* increase was that of the experiment E1 (+C+L+S) where all factors were present. In Fig. 2 it can be observed that all the experiments with CO_2 presence increased their *TL*, and in the experiments without CO_2 presence, the *TL* decreased with the exception of E5 (−C+L+S) where there was presence of light and salt.

The factorial analysis allowed detecting the effects that produced a final increase in the *TL*. Only significant effects according to the ANOVA test are represented in Fig. 3 (numerical results of the ANOVA analysis are showed in Table 4 as Supplementary material). As can be seen in Fig. 3 the three factors presented a direct effect on the *TL*. Moreover, the CO_2 presence was the factor that produced a higher direct effect on the *TL*, followed by the light presence and the salt presence. Several combinations of these factors presented an interaction effect: the combination of CO_2 and salt and the combination of light and salt presence. Results reported by Tang et al. (2011) pointed the direct relation of the CO_2 presence and the *TL* in *S. obliquus* cultures. Ruangsomboon et al. (2013) also determined the positive relation between the salinity of the culture medium and the *TL* content of *Scenedesmus dimorphus*. Even if the results of these authors confirm the salinity effect on the *TL*, their experiments included the start of the cultures at a determined salinity differing with the methodology applied in this study where the salinity was increased at the end of the first stage.

It has to be remarked the *TL* increase in E2 (+C−L+S), that reached 41.9% due to the presence of CO_2 and salt. In this sense, considering a wastewater treatment process based on microalgae technology, keeping the cultures in darkness and in presence of CO_2 and salt should not suppose a considerable increase in overall costs of the process while economic benefits could be increased. As light would not be required to increase the *TL*, the use of photobioreactors would be then avoided. Moreover, it would be possible to couple this step with other process as biomass dewatering via sedimentation.

3.3. Fatty acids

Table 2 shows the results of the analysis of fatty acids of the experimental design in this paper.

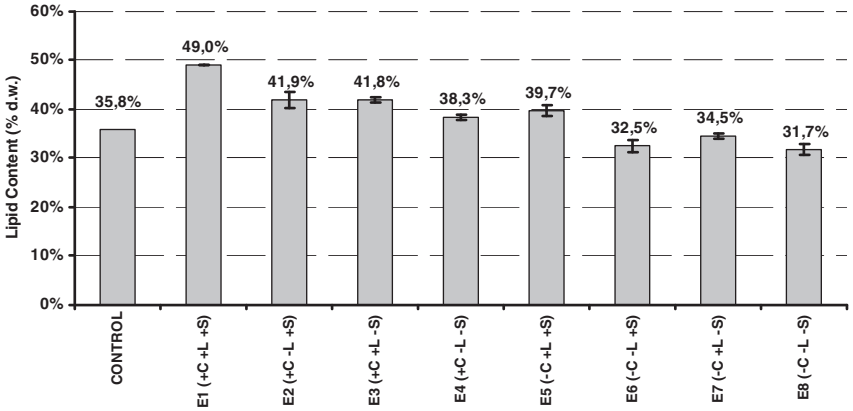

Fig. 2. Lipid content of the biomass. Error bars are confidence intervals ($n = 3$, $\alpha = 0.05$).

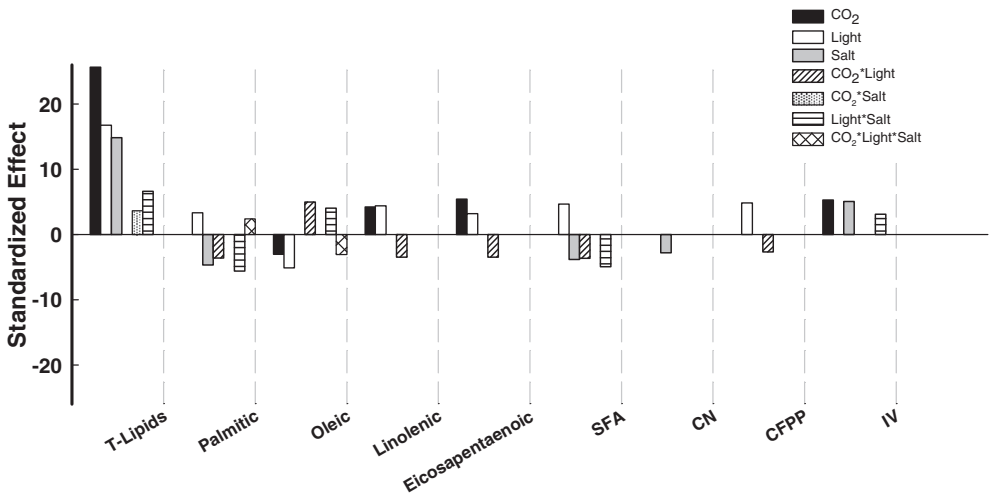

Fig. 3. Standardized effects of CO_2, light and salt and their interactions on the different responses analyzed. Only significant effects ($p > 0.05$) according to ANOVA are represented. *SFA*: saturated fatty acids, *CFPP*: cold filter plugging point, *CN*: cetane number and *IV*: iodine value.

Table 2
Fatty acid relative abundance at the end of the first stage and variations of all experiments at the end of the second stage with respect to their relative content at end of the first stage. Variations above 100% are marked in bold and underlined.

Fatty acid	First stage (%)	E1 (+C+L+S) (%)	E2 (+C−L+S) (%)	E3 (+C+L−S) (%)	E4 (+C−L−S) (%)	E5 (−C+L+S) (%)	E6 (−C−L+S) (%)	E7 (−C+L−S) (%)	E8 (−C−L−S) (%)
C14:0 (myristic)	0.5	0.3	0.4	0.6	0.5	0.4	0.4	0.6	0.3
C16:0 (palmitic)	26.1	17.1	19.2	23.0	21.2	18.5	19.2	25.3	16.1
C16:1 (palmitoleic)	3.3	4.1	3.5	3.5	3.5	4.3	3.3	3.4	2.3
C17:0 (margaric)	1.0	**2.3**	1.3	1.6	1.3	**2.2**	1.2	1.7	0.8
C17:1 (margaroleic)	1.0	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1
C18:0 (estearic)	5.0	3.7	3.8	4.2	3.7	3.9	3.9	4.1	3.7
C18:1 (oleic)	49.2	53.9	53.0	48.6	49.8	52.8	56.0	45.6	63.8
C18:1 cis-11 (vaccenic)	0.2	0.1	0.2	0.3	0.2	0.0	0.0	**0.8**	0.2
C18:2 (linoleic)	5.7	7.0	6.9	7.1	7.3	7.5	6.6	7.2	5.8
C18:3(n-3) (linolenic)	5.5	7.4	7.1	7.4	7.2	7.0	6.1	7.5	4.2
C20:0 (arachic)	0.2	0.2	0.2	0.3	0.3	0.2	0.0	0.3	**0.4**
C20:5(n-3) (eicosapentaenoic)	1.3	1.9	1.8	1.7	1.9	1.8	1.2	1.5	0.8
C20:1 (gadoleic)	1.3	1.2	1.6	1.6	**2.6**	0.8	1.3	1.5	1.3
C22:0 (behenic)	0.3	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0
C22:1(n-9) (erucic)	0.1	**0.3**	**0.5**	**0.4**	**0.3**	0.2	**0.6**	**0.4**	**0.2**
C24:0 (lignoceric)	0.2	0.2	0.0	0.0	0.0	**0.3**	0.0	0.0	0.0

In the first column of Table 2 is presented the *FA* content at the end of the first stage. Following columns are the variations with respect to those contents in the second stage of each experiment. The variations with respect to the first stage were averaged from both replicated experiments. As can be observed, at the end of the first stage, oleic acid (C18:1) with 49.17% of total fatty acids was the most abundant fatty acid followed by palmitic acid (C16:0) with 26.1%. In this sense, Breuer et al. (2013) reported dominance of oleic acid under nitrogen starvation conditions of *S. obliquus* which corroborates the nitrogen starvation of these experiments. The oleic acid accumulation is generally observed in green algae (Griffiths et al., 2012).

The two most abundant fatty acids, oleic and palmitic, presented variations in their content in the second stage. The oleic acid increased its presence in 29.8% when none of the factors were applied and decreased in 7.3% when light was the only factor applied. Besides, the factorial analysis revealed two negative direct effects when the CO_2 or the light was present, two positive interaction effects when the CO_2 and the light were present and when the light and the salt were present (Fig. 3), and also a negative triple interaction effect. Therefore, if the relative content of this fatty acid has to be augmented none of the factors has to be applied on the second stage, while if its content has to be decreased the aging phase has to be conducted under illuminated conditions because the negative effect of light was the strongest. The palmitic acid decreased its content with respect to the first stage in all experiments. The factorial analysis revealed a positive direct effect of light factor while the rest of significant effects were negative.

In regard to the ω-3 fatty acids, the relative content of these *FA* was low. At the end of the first stage, the α-linolenic acid (C18:3ω-3) always presented a higher relative content than the eicosapentaenoic acid (C20:5ω-3) with 5.45% and 1.27%, respectively. On the second stage, this relative abundance increased in most of experiments. The α-linolenic acid increased in all the experiments with the exception of E8 (−C−L−S) where it decreased a 23.2% with respect to the first stage; the averaged increase among the other 7 experiments was about 30%. The eicosapentaenoic acid increased in most of experiments with the exception of E6 (−C−L+S) and E8 (−C−L−S), the averaged increases among the other six experiments was 39.1%. In any case, the CO_2 factor and the light factor presented a positive effect on these two fatty acids when they acted isolated, nevertheless, when both factors acted

Table 3
Variation of the biodiesel critical parameters in each experiment, values were averaged from replicated experiments.

	Cetane number	Cold filter plugging point (°C)	Iodine value (g iodine/100 g)
First stage	58	−2.4	81
E1 (+C+L+S)	55	−2.4	96
E2 (+C−L+S)	56	−2.9	94
E3 (+C+L−S)	56	−1.8	91
E4 (+C−L−S)	56	−3.0	93
E5 (−C+L+S)	56	−1.7	94
E6 (−C−L+S)	56	−4.4	89
E7 (−C+L−S)	57	−1.2	86
E8 (−C−L−S)	56	−4.5	87

together the interaction effect was negative and thus the relative abundance of the ω-3 fatty acids decreased. This implies that the possible strategies to increase the content in these ω-3 fatty acids must take into account only one of these two factors. It has been reported that several microalgae strains can be candidate sources of ω-3 *FA* (Adarme-Vega et al., 2012; Zhou et al., 2012) proposed that microalgae from wastewater treatment with similar ω-3 content than that obtained here could be used as animal feeding.

The unsaturation of *FA* varied with respect to the first stage. In Table 2, the concentration of unsaturated *FA* increased in all the experiments at the second stage. The concentration of monounsaturated *FA* (*MUFA*) increases with the exception of E7 (−C+L−S) and E3 (+C+L−S). Only in E8 (−C−L−S) the concentration of polyunsaturated *FA* (*PUFA*) decreased.

The factorial analysis revealed that the saturation of the *FA* presented a direct positive effect (light presence) and several negative effects, the first was a direct effect with the salt, the second was an interaction effect with the CO_2 and the light, the third was an interaction effect with the CO_2 and the salt and the fourth was an interaction effect with the light and the salt. It has been previously reported that high light conditions induced *FA* saturation in *Dunaliella* sp. cultures. Lee et al. (2014) and Hu et al. (2008) described that high light intensity decreases polar lipid content and it increases the concentration of neutral storage lipids.

3.4. Proprieties of biodiesel according to S. obliquus fatty acid profile

According to Schenk et al. (2008) the ideal mix of fatty acids for biodiesel would present a ratio of 5:4:1 of C16:1, C18:1 and C14:0 fatty acids. Results would provide a biodiesel that will vary significantly from that ratio, as the control experiment presented a ratio of 6:93:1. The great abundance of C18:1 in all experiments (around 50%) could make necessary the addition of C16:1 and C:14 to obtain the ideal mix proportion. Nevertheless, Cha et al. (2011) stated that the ideal mix proposed by Schenk et al. (2008) is not practical because C16:1 and C14:0 are not common in most microalgae oils, and they recommend blending different microalgal oils. Therefore, the models developed to predict the critical biodiesel properties from the fatty acid composition of the oil according to EN 14214 have been used to study the properties of the biodiesel from the experiments (see Table 3).

The *CN*, related to the ignition delay time and combustion quality, increases with increasing chain length of the fatty acids and increasing saturation. In all cases all experiments satisfied the UN 14214 normative ($CN > 51$). Even if the factorial analysis revealed a negative interaction effect related to the salinity increase it should not to be taken into account due to the low adjusted R^2 (0.36) of the ANOVA analysis (Table 4).

The *CFPP* indicates the cold flow property of biodiesel and presents a lower value in relation to the presence of unsaturated fatty acids. The EN 14214 standard does not mention a low-temperature parameter in its list of specifications; however, it discusses the use of a low-temperature filterability test, the cold filter plugging point (*CFPP*). EN 14214 specifies a range of grades with temperature limits depending on climate conditions (Knothe, 2005). The *CFPP* of the first stage was 2.4 °C, satisfying grade A requirements of the EN 14214 ($CFPP < 5$ °C); moreover, all *CFPP* values in the second stage were below 0 °C as the concentration of unsaturated *FA* increased in all experiments, the lowest *CFPP* was −4.5 °C (E8 (−C−L−S)), therefore, all experiments at the end of the second stage satisfied grade A and B requirements of the EN 14214. The factorial analysis revealed a positive direct effect related with the light and one negative interaction effect related to the CO_2 and light presence. Therefore, light must be avoided in order to decrease CFPP or light would be applied in combination with CO_2.

The *IV* refers to the tendency of biodiesel to react with oxygen and it is a measure of total unsaturation within a mixture of fatty acids. All experiments satisfied the requirements of the EN 14214 (<120 g I_2/100 g) presenting values between 80 and 95 g iodine/100 g. The factorial analysis revealed three positive effects, the direct effect of the CO_2, the direct effect of the salinity increase and the interaction effect of the light and salinity effect. Therefore, none of these factors or factor combination should be applied as they will increase the *IV* of the oil.

Also, the EN 14214 biodiesel standard, limits the contents of linolenic acid (C18:3) and fatty acids of more than four double bonds to be less than 12% and 1% (w/w), respectively. The first requirement is widely satisfied as the higher linolenic acid concentration did not reach more than 7.45% (E7 (−C+L−S)). The second requirement is not satisfied as the eicosapentaenoic acid (C20:5ω-3) (*EPA*) concentration, with 5 double bounds is higher than 1% in most of experiments except in E8. In this sense, the concentration of *EPA* at the end of the first stage was 1.27% while the highest value was 1.93% of E1 (+C+L+S). Therefore, the use of oil from *S. obliquus*, cultured under these or similar conditions as unique source of biodiesel, would not be feasible unless it would be blended with other low *EPA* oil.

Nevertheless, as the ω-3 *FA* are high-value products, it would also be possible to selectively extract the ω-3 *FA* content from the oil previously to the transesterification process. In this sense, there are different methods to extract and concentrate ω-3 *FA* as enzymatic concentration, urea inclusion complexation, low-temperature fractional crystallization, liquid–liquid extraction, supercritical fluid extraction and ionic liquid extraction (Cheong et al., 2011).

Even if the application of different factors to the second stage implied variations in the critical properties of the biodiesel, these variations did not imply great changes in the quality of *FA* for the biodiesel. It is then recommended to focus the efforts in the increase of the *TL*, rather than in the modification of the *FA* profile regarding at the biodiesel properties.

4. Conclusion

Microalgae biomass from wastewater treatment systems can be upgraded in a second cultivation stage to increase the lipid content. Extending the stationary growth phase of wastewater cultured *S. obliquus* increased its lipid content until 41.8% in nitrogen and phosphorus starvation and with light and CO_2 presence. A similar result was obtained without light and with a salinity increase (41.9%) avoiding therefore the use of a photobioreactor in the upgrade stage. Oil from *S. obliquus* can be used as biodiesel

source according to EU standard excepting for the ω-3 eicosapentaenoic acid content which slightly exceeded the maximum 1%.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.01.018.

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